Ganglioside GM3 Promotes Carcinoma Cell Proliferation via Urokinase Plasminogen Activator-Induced Extracellular Signal-Regulated Kinase-Independent p70S6 Kinase Signaling

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Overexpression of NeuAcα2-3Galβ1-4Glcβ1-Cer (GM3), a major ganglioside of cutaneous tumor cell membranes, inhibits ligand-dependent and ligand-independent activation of the epidermal growth factor (EGF) receptor in normal and neoplastic epithelial cells. This leads to the suppression of Ras/extracellular signal-regulated kinase (ERK) activation and, in the presence of EGF or fibronectin, inhibits cell proliferation. However, some tumor cells show increased levels of GM3, and vaccines that target GM3 can inhibit the growth of neoplastic cells *in vivo*, especially melanomas. We report that in the presence of urokinase plasminogen activator (uPA), overexpression of GM3 paradoxically increases the proliferation of carcinoma cells by augmenting ERK-independent p70S6 kinase activation. Functional blockade of uPA receptor (uPAR) or inhibition of p70S6 kinase, but not inhibition of Ras/ERK signaling, suppresses this GM3-induced stimulation of cell proliferation. The ERK-independent activation of p70S6 kinase involves phosphorylation at threonine-389, threonine-421/serine-424, and serine-411 sites with intermediate phosphatidylinositol 3 kinase and protein kinase C-ζ activation. These studies implicate gangliosides as enhancers of uPAR-related signaling and suggest that the response to GM3 depends on the local concentration of uPA. Therapeutic modalities that target or supplement gangliosides may require concomitant treatment that suppresses EGFR or uPAR signaling, respectively, to control neoplastic cell proliferation.

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INTRODUCTION

Gangliosides are membrane acidic glycosphingolipids with at least one sialic acid moiety linked to their oligosaccharide chain. The hydrophobic sphingolipid component of gangliosides is anchored within the membrane and the carbohydrate residues extend into the extracellular environment; thus, gangliosides are able to interact with other membrane components or with external agents. Each cell type shows a distinct pattern of ganglioside content, which is altered by neoplasia (Mora *et al.*, 1969; Paller *et al.*, 1992; Hakomori, 2001).

Accumulating evidence indicates that gangliosides influence the activity of several signaling cascades at the membrane level (Wang *et al.*, 2001a, d, 2002a; Hakomori, 2002). Our previous studies have shown that NeuAcα2-3Galβ1-4Glcβ1-Cer (GM3), the predominant ganglioside of neoplastic epithelial cells and melanomas, interacts directly with EGFR (Wang *et al.*, 2001a, c) and inhibits the dimerization of EGFR (Wang *et al.*, 2002a). These interactions inhibit ligand-dependent activation of the EGFR and its downstream Ras/extracellular signal-regulated kinase (ERK) signaling (Wang *et al.*, 2001a, c, 2002a), thus suppressing epithelial cell proliferation in the presence of epidermal growth factor (EGF) (Wang *et al.*, 2001a). Overexpression of GM3 is also able to suppress fibronectin-induced EGFR and Ras/ERK signaling through inhibition of the crosstalk between EGFR and the $\alpha5\beta1$ integrin (Wang *et al.*, 2003a).

The clearly demonstrated inhibitory effects of GM3 on cell proliferation of many cell types suggest that functional depletion of GM3 would stimulate cancer cell growth, whereas therapies that augment GM3 would lead to suppression. Although overexpression of ganglioside-specific sialidase and resultant reduction of GM3 content have been

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Abbreviations: EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GM3, NeuAcα2-3Galβ1-4Glcβ1-Cer; mTOR, mammalian target of rapamycin; PCNA, proliferating cell nuclear antigen ; PI3K, phosphatidylinositol 3 kinase; PKC, protein kinase C; SSIA, SCC12 cells stably transfected with sialidase cDNA; TLC, thin-layer chromatography uPA, urokinase-type plasminogen activator; uPAR, uPA receptor

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seen in some tumors (Kakugawa *et al.*, 2002), low levels of sialidase and increases in GM3 have been found in highly metastatic variants of colon adenocarcinomas (Sawada *et al.*, 2002). Sialidase overexpression in colon adenocarcinoma cells (Sawada *et al.*, 2002) and melanoma cells (Tokuyama *et al.*, 1997) retards cell growth and metastasis. In addition, decreased synthesis of gangliosides by treatment with an inhibitor of glucosylceramide synthesis inhibits melanoma tumor growth in a mouse model (Weiss *et al.*, 2003). Most importantly, gangliosides (such as GM3) have been targeted increasingly for antitumor vaccine development (Bitton *et al.*, 2002; Fernandez *et al.*, 2003; Guthmann *et al.*, 2004) and monoclonal antibody therapy (Irie *et al.*, 2004).

Urokinase plasminogen activator (uPA), a serine protease, is able to bind to its cell membrane receptor, uPAR, and thereby initiates signal-transduction events that lead to remodeling of extracellular matrices, cell migration, invasion, and proliferation (Ossowski and Aguirre-Ghiso, 2000; Jo *et al.*, 2003). A wide variety of human tumors, including melanomas, cutaneous carcinomas, and neoplasms of the breast, lung, bladder, colon, liver, pleura, pancreas, and brain, show increased expression and/or activation of uPA and uPAR (Rosenthal *et al.*, 1998; Blasi and Carmeliet, 2002; Rofstad *et al.*, 2004). These increases have correlated with accelerated cell growth and a poor prognosis. The uPAR is not a transmembrane protein, but is tethered to the cell surface through a glycosylphosphatidylinositol linkage. Therefore, the initiation of signal transduction depends upon its association with transmembrane proteins, including members of the integrin family (Kugler *et al.*, 2003), chemotactic receptors (Resnati *et al.*, 2002), and receptor tyrosine kinases, such as the EGFR (Liu *et al.*, 2002). Although the association of uPAR with these proteins is well documented, little is known about the underlying molecular mechanisms and factors that modulate the uPAR signaling response.

Recent studies have demonstrated that uPAR and ganglioside GM3 cosegregate in membrane rafts (Gomez-Mouton *et al.*, 2001), suggesting that GM3 may participate in the regulation of uPAR signaling. We examined the effect of GM3 elevation on uPAR signaling-induced carcinoma cell proliferation. Surprisingly, we found that GM3 overexpression in the presence of uPA augments, rather than suppresses, uPAR signaling-induced cell proliferation. The mechanism of this novel effect of GM3 appears to involve ERK-independent activation of the 70 kDa ribosomal S6 (p70S6) kinase, a serine/threonine protein kinase that is known to stimulate cell proliferation and survival (Dufner and Thomas, 1999). Activation of protein kinase C (PKC)-ζ and phosphatidylinositol 3 kinase (PI3K) (Weng *et al.*, 1995; Pullen and Thomas, 1997; Romanelli *et al.*, 1999) is required for the uPA-induced stimulation of p70S6 kinase by GM3.

RESULTS

GM3 overexpression stimulates carcinoma cell proliferation

Previous work from our laboratory has demonstrated that elevated expression of GM3 inhibits SCC12 cell proliferation in response to EGF (Wang *et al.*, 2001a) or fibronectin (Wang *et al.*, 2003a). In contrast, increased GM3 levels make these cells much more responsive to the stimulation by uPA (Figure 1). After 24 hours of treatment with uPA, augmentation of GM3 by genetic or biochemical manipulation increases the expression of proliferating cell nuclear antigen (PCNA), a marker of proliferating cells (Figure 1a, top row, lanes 3 and 6) and BrdU incorporation (Figure 1b, bars 3 and 5) relative to control cells (Figure 1a, top row, lanes 1, 2, 4, and 5; Figure 1b, bars 1, 2, and 4). In the presence of either EGF (Figure 1a, middle row) or fibronectin (Figure 1a, bottom row), GM3 overexpression diminishes PCNA expression. In the absence of supplemental uPA, EGF, or fibronectin, PCNA expression and BrdU incorporation are not detected (not shown). The expression of GM3 after these manipu-

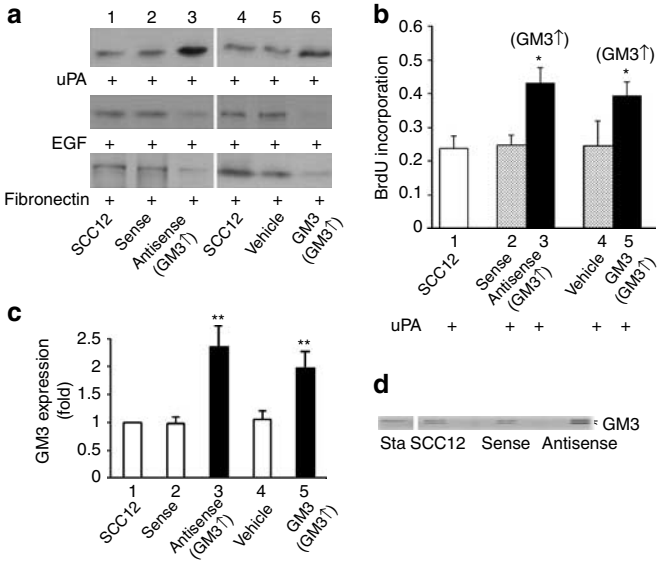

Figure 1. GM3 overexpression stimulates uPA-induced PCNA expression and BrdU incorporation. GM3 expression in SCC12 cells was increased by treatment with either antisense oligomers to block the synthesis of downstream gangliosides of GM3 (Wang *et al.*, 2002a) or pharmacological addition of purified GM3 (Wang *et al.*, 2001a). (**a**) After starvation of serum and growth factors, cells were stimulated with 10 nM uPA (top row), 100 ng/ml EGF (middle row), or 10 μg/ml fibronectin (bottom row) for 24 hours. Eight microgram of total protein from the whole-cell lysate was applied to a 12% SDS-PAGE mini-gel and probed with anti-PCNA antibody; lanes 1 and 4, parental SCC12 cells; lane 2, cells treated with sense oligomers to serve as a control (sense); lane 3, cells treated with antisense oligomers to block the GM3 metabolism (antisense); lane 5, cells treated with vehicle, DMSO, as a control for GM3-treated cells (vehicle); and lane 6, pharmacological addition of GM3 (GM3). (**b**) BrdU incorporation was measured in cells prepared as indicated. The figure represents the means ± SD of studies from three different experiments. The changes in GM3 expression are shown in parentheses; bar 1, parental SCC12 cells; bar 2, cells treated with sense oligomers to serve as a control (sense); bar 3, cells treated with antisense oligomers to block the GM3 metabolism (antisense); bar 4, cells treated with vehicle, DMSO, as a control for GM3-treated cells (vehicle); and bar 5, pharmacological addition of GM3 (GM3). The expression of GM3 after genetic manipulation (increased 2.35-fold, bar 3) or pharmacological addition (increased 1.98-fold, bar 5) was detected by (**c**) ganglioside ELISA (Wang *et al.*, 2002b) and (**d**) TLC immunostaining (Wang *et al.*, 2002a); Sta = ganglioside standard (*$P<0.05$; **$P<0.01$).

lations was increased 2- to 2.5-fold as detected by ganglioside ELISA (Figure 1c; Wang *et al.*, 2002b) and by thin-layer chromatography (TLC) immunostaining (Figure 1d; Wang *et al.*, 2002a, b).

GM3 overexpression triggers uPA-induced p70S6 kinase activation by an ERK-independent pathway

The decreased SCC12 cell proliferation that results from GM3 overexpression in the presence of EGF or fibronectin correlates with the impaired activation of ERK (Wang *et al.*, 2001a, 2003a, b). To address the requirement for ERK activation in uPA-induced cell proliferation when GM3 expression is altered, ERK phosphorylation and activity were assessed. As with cells stimulated with EGF or fibronectin, uPA fails to induce either ERK phosphorylation (Figure 2a, middle row) or activation (Figure 2a, bottom row) when GM3 is elevated (lane 3). To screen for the potential uPA-induced signaling pathways that might be impacted by GM3 overexpression, tyrosine and serine/threonine phosphorylation of the total protein from the whole-cell lysate was assessed. GM3 overexpression results in decreased tyrosine phosphorylation of a band at approximately 170 kDa (presumed to be EGFR), but increases serine/threonine phosphorylation at a band of approximately 70 kDa (data not shown). We hypothesized that this 70 kDa band represented increased serine/threonine phosphorylation of p70S6 kinase, a kinase known to induce cell survival and cell proliferation (Hirata and Kiuchi, 2003).

Kinase assays of uPA-treated cells with increased GM3 expression show significant increases in p70S6 kinase activity (Figure 2b, bars 4 and 7), in comparison with controls (Figure 2b, bars 1, 3, and 6). Blockade of ERK activity does not affect the increased p70S6 kinase activity in cells with overexpressed GM3 (Figure 2b, bars 5 and 8).

Serine and threonine phosphorylation of the p70S6 kinase is increased by GM3 overexpression, even in the presence of blockade of ERK signaling by PD98059 (Figure 2c, lane 3, middle and bottom rows), when compared to control cells (parental SCC12 cells and sense oligomer-treated cells) (Figure 2c, lanes 1 and 2).

In the absence of uPA, no ERK phosphorylation and activity (Figure 2a, lanes 4–6, middle and bottom rows), p70S6 kinase activity (Figure 2b, bars 9–16), or p70S6 kinase phosphorylation (Figure 2c, lanes 4–6, middle and bottom rows) is detected. Modulation of GM3 expression does not affect either ERK or p70S6 kinase expression (not shown).

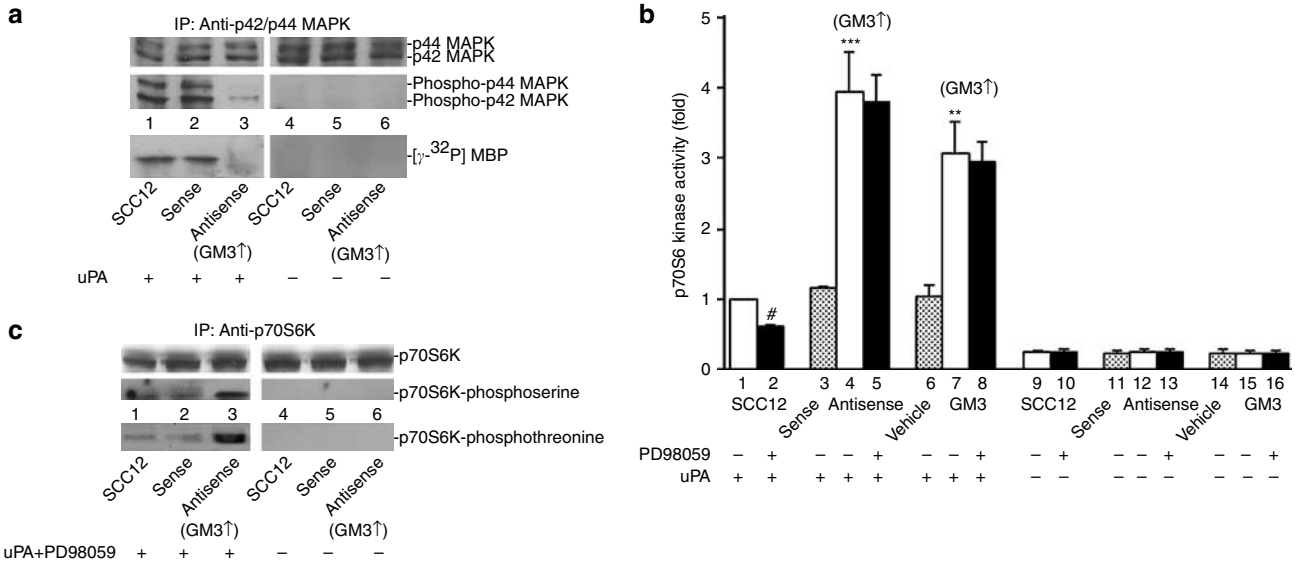

Figure 2. Overexpression of GM3 in the presence of uPA inhibits ERK activation, but stimulates ERK-independent p70S6 kinase activation. Cells were prepared as indicated in Figure 1a. (**a**) ERK and (**b**, **c**) p70S6 kinase were immunoprecipitated from the whole-cell lysate as described in Materials and Methods. Equal loading of the immunoprecipitated kinases was confirmed by immunoblotting with either (**a**, top row) anti-ERK antibody or (**c**, top row) anti-p70S6 kinase antibody . (**a**) Using immunoprecipitated ERK, the effect of GM3 on the phosphorylation of ERK was detected with anti-phospho-ERK antibody (middle row). The activity of this kinase was measured using immunoprecipitated ERK to incubate with its substrate in the presence of [γ-^{32}P]ATP as described in Materials and Methods (bottom row). Lane 1, parental SCC12 cells; lane 2, cells treated with sense oligomers to serve as a control (sense); lane 3, cells treated with antisense oligomers to block the GM3 metabolism (antisense); and lanes 4–6, cells are same as lane 1–3, but without uPA stimulation. (**b**) Using immunoprecipitated p70S6 kinase, the effect of GM3 overexpression on the activity of p70S6 kinase was detected in the presence or absence of PD98059, an ERK inhibitor; bars 1–2, parental SCC12 cells treated without or with PD98059; bar 3, sense oligomer-treated cells (sense); bars 4–5, antisense oligomer-treated cells in the absence or presence of PD98059 (antisense); bar 6, vehicle DMSO-treated cells (DMSO); bars 7–8, cells with pharmacological addition of GM3 in the absence or presence of PD98059 (GM3); and bars 9–16, cells are same as bar 1–8 but without uPA stimulation. (**c**) The phosphorylation of p70S6 kinase was detected using anti-phosphoserine (middle row) or anti-phosphothreonine antibodies (bottom row); lane 1, parental SCC12 cells; lane 2, cells treated with sense oligomers to serve as a control (sense); lane 3, cells treated with antisense oligomers to block the GM3 metabolism (antisense); and lanes 4–6, cells are same as lane 1–3, but without uPA stimulation. Data from the p70S6 kinase assay are presented as fold increase from parental SCC12 cell controls in the absence of inhibitors ($n = 3$, means $\pm$ SD). The figures are representative of all cell lines studied (**$P < 0.01$; ***$P < 0.001$). *P*-values reflect the comparison of cells with increased GM3 with their respective control cells; $^{\#}P < 0.05$ of SCC12 cells with ERK inhibition (solid bar) *versus* no inhibition (open bar).

To further examine the contribution of ERK signaling and p70S6 kinase signaling to uPA-stimulated cell proliferation in the presence of elevated GM3, we also employed GM3-depleted cells. GM3 was depleted by treatment of cells with threo-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol HCl (PPPP) (a chemical reagent to block the synthesis of GM3 precursor) or by stable transfection of a human ganglioside-specific plasma membrane sialidase (to cleave the sialic acid residue from GM3 and render GM3 non-functional). The depletion of GM3 in PPPP-treated cells and sialidase-overexpressing cells (SSIA) was confirmed by TLC immunostaining (Figure 3a). Ceramide, a bioactive molecule, is a precursor of GM3 and is known to affect cell signaling. To clarify the effect of GM3 modulation on ceramide expression, TLC immunostaining was performed. Modulation of GM3 expression by treatment of cells with PPPP does not lead to the accumulation of ceramide (Figure 3b) (Abe *et al.*, 1995).

In the presence of elevated GM3, cells were treated with either PD98059, an inhibitor of ERK, or rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), and p70S6 kinase, which is the downstream signaling molecule of mTOR. Inhibition of p70S6 kinase activity (Figure 3c, lane 7), but not ERK activity (Figure 3c, lane 6), eradicates the uPA-induced PCNA expression triggered by increased GM3. In contrast, uPA-induced PCNA expression in control cells (cells without increased GM3) could be prevented by inhibiting ERK activity (Figure 3c, lane 2), but not p70S6 kinase activity (Figure 3c, lane 3). Activity assays showed that rapamycin (Figure 3d, bar 7), but not PD98059 (Figure 3d, bar 6), interferes with the p70S6 kinase activity triggered by GM3 overexpression in comparison with cells treated without inhibitor (Figure 3d, bar 5) ($P<0.001$). This result indicates that uPA-induced cell proliferation in cells with elevated GM3 depends on p70S6 kinase activation, not ERK signaling.

Depletion of GM3 also significantly increases the activity of p70S6 kinase (Figure 3d, bars 9 and 13). Treatment with rapamycin to block p70S6 kinase activation (Figure 3d, bars 11 and 15) or with PD98059 to block ERK signaling (Figure 3d, bars 10 and 14) eliminates the increase in p70S6 kinase activity. However, only blockade of ERK signaling eradicates the expression of PCNA in cells with depleted gangliosides (Figure 3c, lanes 10 and 14).

Given the contribution of ERK signaling to the increased p70S6 kinase activity of GM3 depletion (Figure 3d, bars 10 and 14) and in an effort to focus on cell function modulated by ERK-independent p70S6 kinase activation, all additional studies were performed in the presence of both uPA and the inhibitor of ERK, PD98059.

The uPA-stimulated p70S6 kinase activation and PCNA expression in the presence of elevated GM3 require uPAR signaling

Neutralization of the uPAR bioactivity with anti-human uPAR antibody (R&D Systems Inc., Minneapolis, MN) blocks ERK-independent PCNA expression in cells with elevated GM3 (Figure 4a, lane 9) in comparison with untreated GM3-overexpressing cells (Figure 4a, lanes 7 and 8). Anti-uPAR antibody also profoundly inhibits p70S6 kinase activation triggered by elevated GM3 in the presence of uPA and

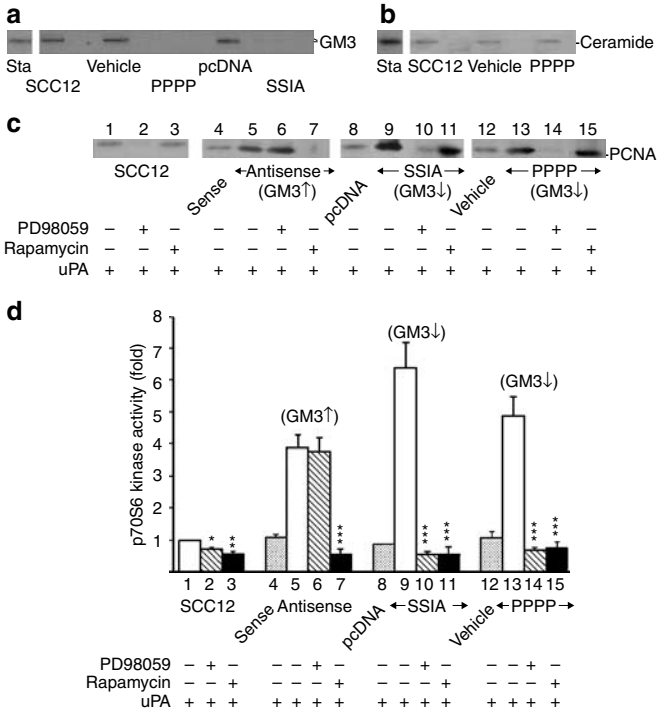

Figure 3. Rapamycin, but not PD98059, blocks the uPA stimulation of PCNA expression in cells with elevated GM3. (**a**) GM3 was depleted either by stable transfection of cells with a human plasma ganglioside-specific sialidase (SSIA) to render GM3 non-functional or by treatment of cells with 2 μM PPPP for 5 days to block the synthesis of GM3 precursor, lactosylceramide. (**b**) Treatment with PPPP did not affect ceramide expression. Gangliosides and ceramide were detected by TLC immunostaining (Wang *et al.*, 2002a). Cells with or without GM3 modulation were treated with 100 μM of PD98059, a specific inhibitor of ERK, or 100 ng/ml of rapamycin (Chung *et al.*, 1992), an inhibitor to mTOR and p70S6 kinase (a downstream signaling molecule of mTOR), for 12 hours and then stimulated with uPA for 10 minutes. (**c**) Eight microgram of total protein from the whole-cell lysates was applied to a 12% SDS-PAGE mini-gel and probed with anti-PCNA antibody; lanes 1–3, parental SCC12 cells treated with or without PD98059 or rapamycin; lane 4, sense oligomer-treated cells (sense); lanes 5–7, antisense oligomer-treated cells in the absence of presence of either PD98059 or rapamycin (antisense); lane 8, pcDNA-transfected mock control for SSIA cells (pcDNA); lanes 9–11, SSIA cells treated without or with PD98059 or rapamycin; lane 12, vehicle DMSO-treated cells (vehicle); and lanes 13–15, cells treated with PPPP in the absence or presence of PD98059 or rapamycin. p70S6 kinase was immunoprecipitated from the whole-cell lysates and incubated with S6 kinase peptide in the presence of [γ-^{32}P]ATP as indicated in Materials and Methods. (**d**) The activity of p70S6 kinase ($n=3$, mean$\pm$SD) is presented as fold increase above parental SCC12 cells treated without inhibitors ; bars 1–3, parental SCC12 cells treated with or without PD98059 or rapamycin; bar 4, sense oligomer-treated cells (sense); bars 5–7, antisense oligomer-treated cells in the absence of presence of either PD98059 or rapamycin (antisense); bar 8, pcDNA-transfected mock control for SSIA cells; bars 9–11, SSIA cells treated without or with PD98059 or rapamycin; bar 12, vehicle DMSO-treated cells (vehicle); and bars 13–15, cells treated with PPPP in the absence or presence of PD98059 or rapamycin. The figures are representative of all cell lines studied (*$P<$ 0.05; **$P<0.01$; ***$P<0.001$). *P*-values compare the hatched or solid bars with the open bars. Sta = ganglioside standard; pcDNA = vector control for SSIA; and SSIA = cells stably transfected with human ganglioside-specific plasma membrane sialidase.

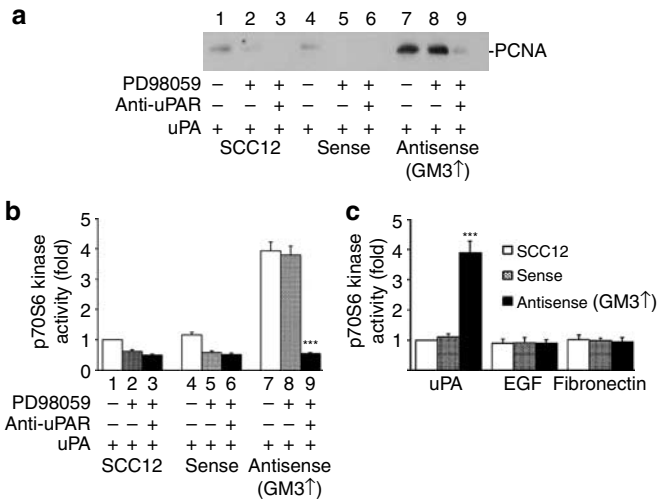

Figure 4. The stimulation of p70S6 kinase activity by GM3 overexpression requires uPAR signaling. GM3 expression in SCC12 cells was endogenously modulated as described in Materials and Methods. The expression of GM3 was detected by ganglioside ELISA or TLC immunostaining as shown in Figures 1 and 3. (**a**) Starved cells were incubated in DMEM/F12 medium containing 0.6% BSA with or without 1 μg/ml of anti-uPAR neutralization antibody in the presence or absence of PD98059 for 24 hours before the stimulation with uPA. After stimulation with 10 nM uPA, cells were lysed, and (**a**) PCNA expression or (**b**) p70S6 kinase activity was detected as described; lane or bar 1, parental control cells (*SCC12*); lane or bar 2, PD98059-treated parental control cells; lane or bar 3, PD98059- and anti-uPAR antibody-treated parental control cells; lane or bar 4, negative control cells (sense); lane or bar 5, PD98059-treated negative control cells; lane or bar 6, PD98059- and anti-uPAR antibody-treated negative control cells; lane or bar 7, cells with increased GM3 (antisense); lane or bar 8, PD98059-treated GM3-overexpressing cells; and lane or bar 9, PD98059- and anti-uPAR antibody-treated GM3-overexpressing cells. (**c**) Starved cells were stimulated with 10 nM uPA, 100 ng/ml EGF or 10 μg/ml fibronectin (Wang *et al.*, 2003a) for 10 minutes. p70S6 kinase was immunoprecipitated from cells and the activity of p70S6 kinase was measured as described above (***$P<0.001$).

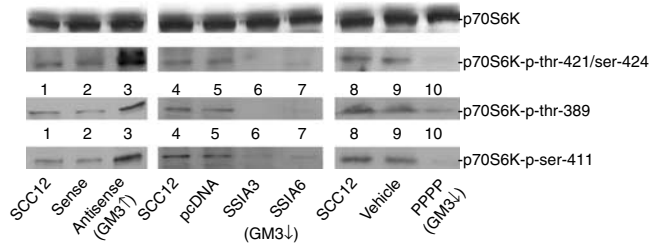

Figure 5. uPA stimulates ERK-independent p70S6 kinase phosphorylation in cells with elevated GM3, but not in cells without GM3. Cells prepared as described in Figure 3. To study ERK-independent p70S6 kinase activation, cells were treated with 100 μM of PD98059, a specific inhibitor of ERK, for 12 hours, and then stimulated with uPA for 10 minutes. p70S6 kinase was immunoprecipitated from cells as described in Materials and Methods. An aliquot of the immunoprecipitated p70S6 kinase was applied onto a 10% SDS-PAGE mini-gel and probed with antibody directed against the p70S6 kinase (top row), p70S6 kinase phosphothreonine 421/serine 424 (second row), p70S6 kinase phosphothreonine 389 (third row), or p70S6 kinase phosphoserine 411 site (bottom row). Lanes 1, 4, and 8, parental control cells (SCC12); lane 2, sense oligomer-treated control cells (sense); lane 3, GM3-overexpressing cells (antisense); lane 5, pcDNA-transfected mock control cells; lanes 6–7, sialidase-overexpressing cells with depleted GM3 (SSIA3 and SSIA 6 clones); lane 9, vehicle DMSO-treated cells (vehicle); and lane 10, PPPP-treated cells with depleted GM3.

PD98059 (Figure 4b, bar 9 *versus* 8, $P<0.001$). Neither EGF nor fibronectin affects the activation of p70S6 kinase in cells with increased GM3 (Figure 4c, solid bars). These data indicate that p70S6 kinase activation triggered by GM3 depends on uPAR signaling.

Activation of uPAR in cells that overexpress GM3 induces ERK-independent phosphorylation of p70S6 kinase at threonine 421/serine 424, threonine 389, and serine 411 sites (Figure 5, lane 3 of the second, third and bottom rows) in comparison with control cells (lanes 1 and 2). GM3 depletion prevents p70S6 kinase phosphorylation at all these sites when ERK activity was inhibited (Figure 5, lanes 6, 7, and 10 of the second, third and bottom rows) compared with controls (lanes 4, 5, 8, and 9).

Activation of PI3K or PKC-ζ phosphorylates ERK-independent p70S6 kinase at specific sites and enhances p70S6 kinase activity in cells with elevated GM3

Several signaling pathways are known to modulate the activation of p70S6 kinase, including PI3K, mTOR, and PKC signaling (Chung *et al.*, 1992, 1994; Pullen and Thomas, 1997; Romanelli *et al.*, 1999). To address the possible role of PKC and PI3K signaling in the activation of p70S6 kinase by uPAR signaling in the presence of elevated GM3, cells were treated with inhibitors of PKC or PI3K signaling. We have previously found that PKC-α, PKC-δ, PKC-η, PKC-φ, and PKC-ζ are expressed by SCC12 cells (unpublished data). SCC12 cells were treated with inhibitors directed against PKC-α, PKC-δ, PKC-η, PKC-φ, or PKC-ζ, and the effects of these inhibitors on p70S6 kinase activation were observed (data not shown). Of these, only PKC-ζ inhibitor decreases p70S6 kinase phosphorylation and activity (Figure 6a and b), as has previously been demonstrated in human embryonic kidney cells (Romanelli *et al.*, 1999). Treatment of SCC12 cells with a synthetic pseudopeptide of PKC-ζ (SIYRRGARRWRKL, Calbiochem) (Laudanna *et al.*, 1998) dramatically inhibits PKC-ζ activity (Figure 6b, inset) and, in the presence of supplemental uPA and elevated GM3, dramatically reduces p70S6 kinase phosphorylation at both the threonine 421/serine 424 and serine 411 sites (Figure 6a, lane 6 of the top and middle rows). Blockade of PI3K activity with either 20 μM of LY294002 or 100 nM of wortmannin significantly inhibits the p70S6 kinase phosphorylation at both threonine 421/serine 424 and threonine 389 sites (Figure 6a, lanes 3 and 4 of the top and bottom rows). The decrease in p70S6 kinase phosphorylation at these specific sites by PI3K and PKC-ζ inhibitors correlates with the reduction in p70S6 kinase activity (Figure 6b). Inhibition of either PI3K (Figure 6b, bar 4) or PKC-ζ (Figure 6b, bar 5) alone leads to partial inhibition of p70S6 kinase activation, whereas inhibition of both PI3K and PKC-ζ concurrently reduces p70S6 kinase activities to baseline levels (Figure 6b, bar 6) in comparison with untreated GM3-overexpressing cells (bar 3), parental SCC12 cells (bar 1), and sense oligomer-treated control cells (bar 2).

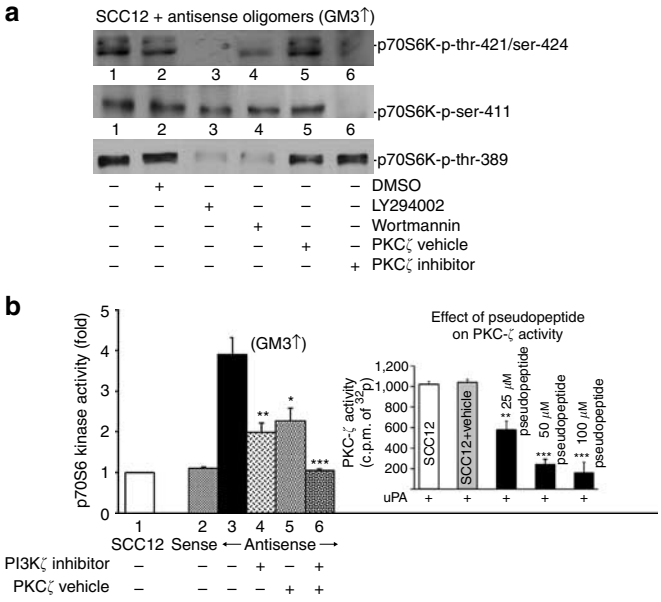

Figure 6. PKC-ζ and PI3K signaling pathways mediate uPA-induced ERK-independent p70S6 kinase activation and phosphorylation at specific sites in the presence of elevated GM3. (**a**) Cells with elevated GM3 prepared as described in Figure 1 were treated with 100 μM of PD98059, a specific inhibitor of ERK, in the presence or absence of 20 μM of LY294002 or 100 nM of wortmannin (both inhibitors of PI3K), 50 μM of the pseudopeptide inhibitor of PKC-ζ, or both LY294002 (20 μM) and pseudopeptide inhibitor of PKC-ζ (50 μM). The vehicle of the PI3K inhibitors or PKC-ζ pseudopeptide was applied as a control. The phosphorylation of p70S6 kinase at different sites was detected as described in Figure 5; lane 1, GM3-overexpressing cells; lane 2, cells treated with DMSO to serve as a vehicle control for PI3K inhibitor-treated cells; lane 3, cells treated with PI3K inhibitor, LY294002; lane 4, cells treated with PI3K inhibitor, wortmannin; lane 5, cells treated with PBS as a vehicle control for cells treated with PKCζ pseudopeptide inhibitor; and lane 6, cells treated with PKCζ pseudopeptide inhibitor. (**b**) Cells were prepared as indicated in Figure 6a. The effect of PI3K and PKC-ζ on p70S6 kinase activity was measured as described in Materials and Methods. The activity of p70S6 kinase is presented as fold increase above parental SCC12 cells treated without inhibitors ($n=3$, mean ± SD) The effect of pseudopeptide inhibitor on uPA-stimulated PKC-ζ activity in normal SCC12 cells was detected by *in vitro* kinase activity assay as described in Materials and Methods and shown as the inset in 6(b) (*$P<0.05$; **$P<0.01$; ***$P<0.001$); bar 1, parental control cells (SCC12); bar 2, sense oligomer-treated control cells (sense); and bars 3–6, GM3-overexpressing cells (antisense) treated without (bar 3) or with PI3K inhibitor, LY294002 (bar 4), PKCζ pseudopeptide inhibitor (bar 5), or both inhibitors (bar 6).

The effect of GM3 overexpression on uPA-induced activation of PI3K and PKC-ζ was analyzed by *in vitro* kinase activity assays (Wang *et al.*, 2002b). uPA stimulates the activation of both PI3K (Figure 7a, lanes 3 and 5 of top row) and PKC-ζ (Figure 7b, solid bars), independent of ERK signaling. Alteration in GM3 expression does not affect the expression of PI3K (Figure 7a, bottom row) or PKC-ζ (not shown).

DISCUSSION

In this report, we have shown that increased levels of ganglioside GM3 in human squamous carcinoma cells activate uPA-induced p70S6 kinase signaling and thereby stimulate carcinoma cell proliferation by an ERK-independent signaling pathway. p70S6 kinase is a ubiquitously expressed serine/threonine protein kinase that phosphorylates the 40S ribosomal protein S6 in response to mitogen stimulation (Dufner and Thomas, 1999). Inactivation of p70S6 kinase is known to suppress cell growth and survival (Lane *et al.*, 1993; Pullen and Thomas, 1997). The activation of p70S6 kinase and stimulation of cell proliferation by elevated GM3 specifically requires the presence of uPA, an activator that is expressed at higher levels in many cancer cells and the activity of which correlates inversely with prognosis. This novel stimulatory effect of GM3 contrasts sharply with the previously demonstrated inhibitory effects of GM3 on squamous carcinoma cell proliferation when the same cells are grown without supplemental uPA, but in the presence of EGF or on a fibronectin matrix (Wang *et al.*, 2001a, 2003a). Activation of p70S6 kinase signaling may be either independent of (Ming *et al.*, 1994) or dependent on (Eguchi *et al.*, 1999) Ras/ERK signaling. Here, we demonstrate that GM3 overexpression augments uPA-induced ERK-independent p70S6 kinase activation, whereas GM3 depletion promotes ERK-dependent p70S6 kinase activation. Furthermore, inhibition of ERK-independent p70S6 kinase, but not of ERK or ERK-dependent p70S6 kinase, eliminates the proliferative effect caused by GM3 overexpression. This implicates GM3 as a membrane adaptor molecule that modulates the effect of ERK signaling on p70S6 kinase activation as well as the effect of p70S6 kinase on cell proliferation.

The binding of uPA to uPAR is well known to cause proteolysis. In addition, binding of uPA to its receptor can activate ERK signaling (Aguirre-Ghiso *et al.*, 2001). This leads to constitutive activation of focal adhesion kinase, PI3K, PKC, EGFR, and mitogen-activated protein kinase and results in uPA/uPAR-dependent tumor growth (Ossowski and Aguirre-Ghiso, 2000; Aguirre-Ghiso *et al.*, 2001). For the first time, we have shown that uPAR activation can also trigger p70S6 kinase activation, either through ERK or ERK-independent pathways, depending on the expression of ganglioside GM3. In the presence of elevated GM3, the stimulatory effects of GM3 on uPA-induced ERK-independent activation of p70S6 kinase signaling and cell proliferation overcome the inhibitory effects of GM3 on ERK signaling-modulated cell proliferation.

Several signaling molecules modulate the activation of p70S6 kinase, among them PI3K, mTOR, and PKC (Chung *et al.*, 1992, 1994; Pullen and Thomas, 1997; Romanelli *et al.*, 1999). The regulation of p70S6 kinase activation is complex and requires phosphorylation at multiple sites for full activation. Our studies also demonstrate that ERK-independent activation of S6 kinase by uPAR signaling involves phosphorylation at threonine 421/serine 424, threonine 389, and serine 411 sites, sites that require activation of PI3K or PKC-ζ for phosphorylation (Eguchi *et al.*, 1999; Romanelli *et al.*, 1999; Zhang *et al.*, 2001). These findings implicate PI3K and PKC-ζ, both known activators of p70S6 kinase, as intermediates in the signaling pathway by which overexpression of GM3 triggers p70S6 kinase activation in the presence of uPA.

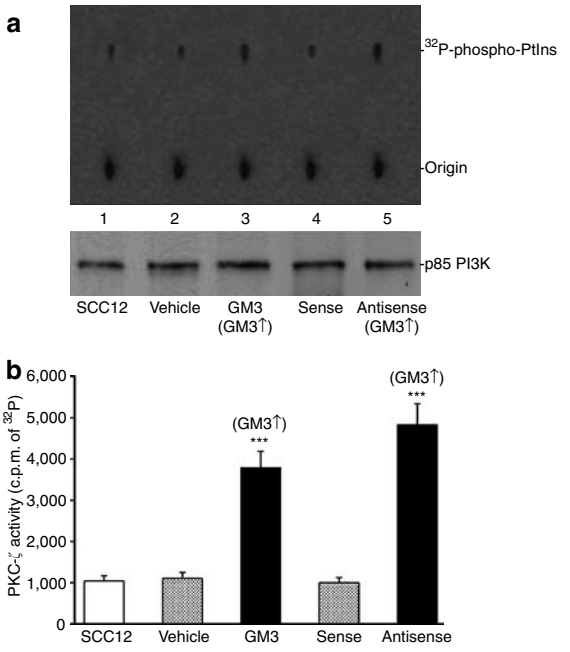

Figure 7. uPA activates PI3K and PKC-ζ in the presence of ERK inhibitors. Cells prepared as described in Figure 1a were treated with the PD98059, an ERK inhibitor, and 10 nM uPA was added for 10 minutes. (**a**) The PI3K activity was measured using the immunoprecipitated p85 subunit of PI3K. After incubation of the immunoprecipitated PI3K with its substrate, phosphatidylinositol, in the presence of [γ-^{32}P]ATP, PI3K activity was detected as described (Wang *et al.*, 2002b); lane 1, parental control (SCC12); lane 2, DMSO-treated vehicle control (vehicle); lane 3, pharmacological addition of GM3 (GM3); lane 4, sense oligomer-treated control (sense); and lane 5, antisense oligomer-treated cells with increased GM3 (antisense). (**b**) The activity of PKC-ζ was analyzed using immunoprecipitated PKC-ζ in the presence of both MBP and [γ-^{32}P]ATP as described in Materials and Methods (***$P<0.001$); bar 1, parental control (SCC12); bar 2, DMSO-treated vehicle control (vehicle); bar 3, pharmacological addition of GM3 (GM3); bar 4, sense oligomer-treated control (sense); and bar 5, antisense oligomer-treated cells with increased GM3 (antisense).

The mechanism by which GM3 associates with uPAR at the membrane level to trigger S6 kinase activation is unclear. Gomez-Mouton *et al.* (2001) have shown the co-segregation of GM3 and uPAR in membrane lipid rafts, sites at which signaling molecules are thought to aggregate to enable communication. We have recently shown that GM3 regulates the ability of uPAR and integrin $\alpha_5\beta_1$ to associate with EGFR and affect EGFR signaling (Wang *et al.*, 2005). GM3 does not affect either the binding of uPA with uPAR or the expression of uPAR (unpublished data). Given the known association of uPAR, integrins ($\alpha_5\beta_1$ and $\alpha v\beta 3$), and caveolin-1 in raft areas (Stahl and Mueller, 1995; Wei *et al.*, 1996, 2005; Hapke *et al.*, 2001; Wang *et al.*, 2005), and the known ability of GM3 to associate with caveolin-1 (Wang *et al.*, 2002a), integrins (Wang *et al.*, 2005) and uPAR (unpublished data), we propose that GM3 modulates uPAR signaling and activates p70S6 kinase through this lipid raft-based complex (see Figure 8 for hypothetical model).

The increased expression of gangliosides, uPA, and uPA receptors in a variety of malignant tumors may provide a milieu in which gangliosides stimulate tumor cell proliferation through augmentation of uPAR signaling. GM3, a predominant ganglioside of neoplastic epithelial cells and melanocytes, has been targeted as a candidate for antiganglioside therapeutic regimens and is also considered as a potential inhibitor of tumor cell growth (Watanabe *et al.*, 2002). Our data suggest that the ability of gangliosides to regulate cell behavior depends greatly on the tumor cell milieu and the presence of growth factors and matrix components, including uPA. These studies provide a rationale for the treatment of malignancies concurrently with either agents that target both GM3 and EGFR signaling pathways, or that increase GM3 membrane content and inhibit uPAR signaling, depending on the tumor cell response to GM3.

MATERIALS AND METHODS

Cells

The human squamous carcinoma cell, SCC12F2 cell line (SCC12) (courtesy of Dr James Rheinwald, Boston, MA), was chosen as the model cell, given the previously shown inhibitory effect of GM3 on proliferation in the presence of EGF or fibronectin (Wang *et al.*, 2001c, 2002a, 2003a) and low basal expression of uPA in this cell line. SCC12 cells were maintained in Dulbecco's modified eagle's medium/F12 (DMEM/F12, 1:1, (v/v)) medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum without antibiotics in 5% CO_2 at 37°C.

Ganglioside expression modulation

Expression of ganglioside by SCC12 cells was modulated by (a) pharmacological addition of purified GM3 (Sigma, St Louis, MO) into cell culture medium as described (Wang *et al.*, 2001a, b, d) to increase cell membrane GM3 content; (b) antisense oligodeoxynucleotide treatment to overexpress ganglioside GM3 by blockade of the synthesis of downstream gangliosides of GM3 (Wang *et al.*, 2002a, 2003a, b); (c) stable transfection of SCC12 cells with a human plasma membrane, ganglioside-specific sialidase cDNA (GenBank accession number AB008185, courtesy of Dr T. Miyagi, Tokyo, Japan), (Wada *et al.*, 1999) to cleave the sialic acid residue from GM3 and render it non-functional (Wang *et al.*, 2001a; Sun *et al.*, 2002); and (d) treatment of cells with 2 μM PPPP (Matreya, Pleasant Gap, PA), which prevents the formation of glucosylceramide, the GM3 precursor (Wang *et al.*, 2002a, 2003b).

Immunoblotting

Immunoblotting was carried out as described (Wang *et al.*, 2001b, d) using immunoprecipitated protein or total protein from the whole-cell lysates and an enhanced chemiluminescence detection system (Perkin-Elmer Life Sciences, Wellesley, MA). Whole-cell lysate was acquired using cells prepared as described (Wang *et al.*, 2002a). In brief, cells with or without modulation of ganglioside expression were treated with or without inhibitors of ERK (100 μM of PD98059, Calbiochem, San Diego, CA), p70S6 kinase (100 ng/ml of rapamycin, Sigma), PI3K (20 μM of LY294002, Calbiochem, or 100 nM of wortmannin, Sigma) or PKC-ζ (50 μM of pseudopeptide, SIYRRGARRWRKL (positions 113–125), Calbiochem) (Laudanna *et al.*, 1998) for 24 hours. Cells were then starved of both serum and growth factors overnight in the presence or absence of inhibitors, and

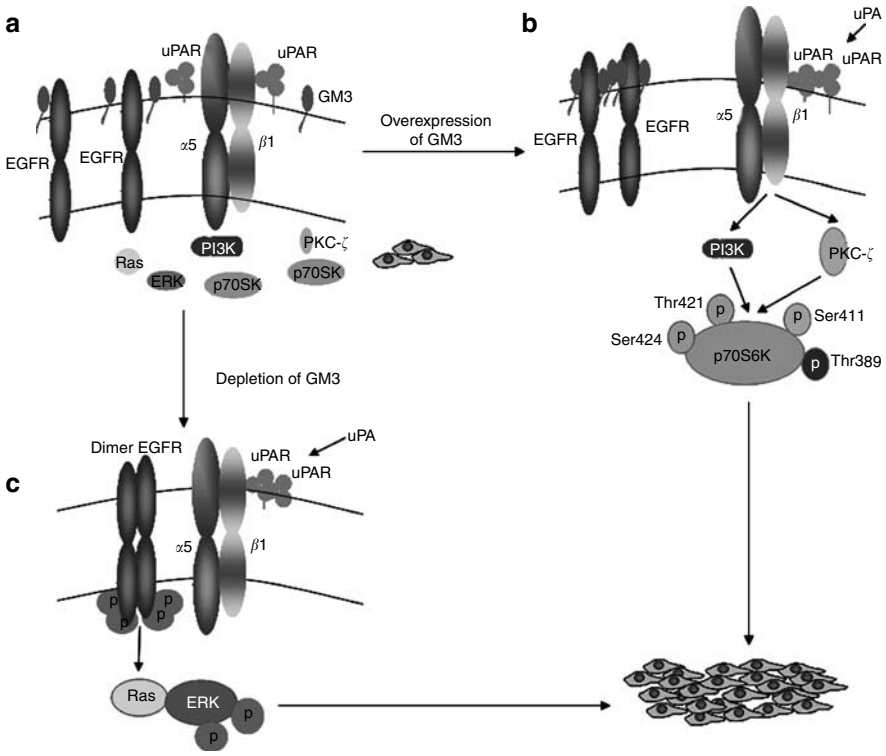

Figure 8. Proposed mechanism of GM3-triggered uPAR activation and cell proliferation. uPAR associates with integrin $\alpha5\beta1$ (Wang *et al.*, 2005) to cross-activate EGFR signaling (Wang *et al.*, 2003a) in the presence of uPA. uPA is able to stimulate cell proliferation via either ERK-dependent or ERK-independent pathways. Cells show increased proliferation in the presence of uPA whether GM3 is increased or decreased. However, the underlying mechanism of increasing cell proliferation is different in the presence of increased GM3 *versus* the absence of GM3. In contrast to (**a**) quiescent control cells, cells that (**b**) overexpress GM3 show activation of PI3K and PKC-ζ, increased p70S6 kinase activity, and cell proliferation, despite suppression of ERK signaling. Cells in which GM3 is (**c**) depleted show activation of ERK signaling, leading to increased cell proliferation that is independent of p70S6 kinase activation.

stimulated with 10 nM uPA (Calbiochem) for 10 minutes to 24 hours before lysis. For immunoblotting, an aliquot of immunoprecipitates or 8–60 μg of total protein from the whole-cell lysate was treated with denatured or undenatured Laemmli buffer as indicated in the figure legends, and loaded onto SDS-PAGE mini-gels. After transfer to polyvinylidene difluoride or nitrocellulose membranes, the separated proteins were detected by immunoblotting with antibodies directed against the PCNA, p70S6 kinase, phospho-p70S6 kinase at Thr-421/Ser-424, Thr-389, or Ser-411sites (Santa Cruz Biotech, Inc., Santa Cruz, CA), phosphoserine, phosphothreonine (CalBiochem), p42/p44 mitogen-activated protein kinase (ERK1/2), and phospho-ERK1/2 (BioSource, Camarillo, CA). Blots using total protein were reprobed as described previously (Wang *et al.*, 2001b) with anti-actin antibody (Santa Cruz Biotechnology Inc.) to confirm equal loading. All blots were repeated in at least three different experiments. When using the immunoprecipitated protein for blotting, the 150 kDa undenatured whole IgG band, 55 kDa denatured heavy chain of the IgG band, and, when detectable, the 25 kDa denatured light chain of the IgG band were cut from the blots for figure presentation.

Immunoprecipitation

Cells were prepared as indicated above and stimulated with or without 10 nM uPA for 10 minutes after starvation of serum and EGF for overnight. Cells were harvested and lysed in cold immunoprecipitation buffer as described previously (Wang *et al.*, 2001b, d). Total protein (1 mg) from the cell lysates was mixed with 5 μg of polyclonal antibody directed against p70S6 kinase (Santa Cruz Biotechnology Inc), ERK1/2, p85 subunit of PI3K, or PKC-ζ and the total reaction volume was adjusted to 1 ml in the immunoprecipitation buffer. After incubation with the antibodies for 2 hours at 4°C, protein A: agarose was added for an additional 30 minutes at 4°C (Wang *et al.*, 2001b).

Kinase activity assays

Kinase activity was assessed using immunoprecipitated p70S6 kinase, ERK1/2, PI3K, or PKC-ζ (Eguchi *et al.*, 1999; Wang *et al.*, 2001a). An S6 kinase peptide (RRRLSSLRA, Santa Cruz Biotech Inc) was selected as a substrate for measuring p70S6 kinase activity. Myelin basic protein (Sigma) was selected as a substrate for determining ERK activity and PKC-ζ activity, and phosphatidylinositol (Sigma) was chosen as a substrate for PI3K activity analysis. p70S6 kinase, ERK1/2, PI3K, or PKC-ζ was immunoprecipitated from the whole-cell lysate with polyclonal antibody directed against p70S6 kinase, ERK1/2, p85 subunit of PI3K, or PKC-ζ, respectively, as described above. Whole-cell lysates were prepared from stimulated starved cells with 10 nM of uPA for 10 minutes. One-third of the immunoprecipitate was boiled in Laemmli buffer and applied to a 12 or 10% SDS-PAGE mini-gel to confirm the purity and equal precipitation of proteins by probing the membrane with anti-p70S6

kinase, anti-ERK1/2, anti-p85 subunit of PI3K, or anti-PKC-ζ antibody. The other two-thirds of the immunoprecipitate was used for kinase assays. For p70S6 kinase activity assays, the reaction was initiated by [γ-^{32}P]ATP (3000 Ci/mmol, Perkin-Elmer Life Sciences) as described previously (Eguchi *et al.*, 1999). PKC-ζ activity assays were performed as described (Wali *et al.*, 2003) by the initiation of the reaction with 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Perkin-Elmer Life Sciences) and 10 μg MBP in a total reaction volume of 50 μl. The γ-^{32}P-labeled product from either the p70S6 kinase activity assay or the PKC-ζ activity assay was measured in a Beckman LS 6000 liquid scintillation counter. Assays for the activity of ERK were performed as described previously (Wang *et al.*, 2001a). The γ-^{32}P-labeled MBP was treated with boiled Laemmli buffer and applied to a 12% SDS-PAGE mini-gel. PI3K activity was measured as described (Wang *et al.*, 2002b). The phosphorylated products from either ERK activity or PI3K activity analysis were visualized by autoradiography with Kodak X-Omat film, developed at −80°C overnight, and quantified by a Storm 800 Fluorescence PhosphorImager. All kinase activity assays were performed three times in triplicate.

BrdU incorporation assays

BrdU incorporation assays were performed to monitor the effect of modulation of GM3 expression on cell proliferation. SCC12 cells were treated with or without either sense or antisense oligodeoxynucleotides to both GM2/GD2 synthase and GD3 synthase, and with or without 140 μM purified GM3 or its vehicle, DMSO. These treated or untreated SCC12 cells were synchronized in the G_0 phase by serum and growth factor starvation for 6 hours in the presence or absence of either oligodeoxynucleotides or purified GM3. Cells were then trypsinized, detached, and plated onto 96-well plates (10^4 cells/well). After incubation in serum- and growth factor-free medium to further synchronize cells for 12–18 hours, 10 nM uPA and 10 μM BrdU were added for another 24 hours. BrdU incorporation was stopped by washing cells with cold PBS (pH 7.6). Cells were then fixed, stained and the absorbance was read at A_{450} nm as per the manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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